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(71) Applicant *(for all designated States except US)*: CROPDESIGN N.V. [BE/BE]; Technologiepark 3, B-9052 Zwijnaarde-Gent (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BURSSENS, Sylvia [BE/BE]; Bosstraat 60, B-9620 Zottegem (BE). VERBRUGGEN, Nathalie [BE/BE]; 53, avenue des Saisons, B-1050 Ixelles (BE). INZÉ, Dirk [BE/BE]; Driesstraat 18, B-9310 Moorsel-Aalst (BE). BEECKMAN, Tom [BE/BE]; Avenskouter 9, B-9820 Merelbeke (BE).

(74) Agent: VOSSIUS & PARTNER; P.O. Box 86 07 67, D-81634 München (DE).

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(57) Abstract

A method for obtaining plants, tolerant to abiotic stress conditions, in particular osmotic stress, is described, by conferring to a plant the capacity to counteract the stress-induced phosphorylation of Cyclin Dependent Kinase (CDK) proteins. Also vectors for conferring the said capacity are provided.

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Stress tolerant plants

The present invention relates to a method for obtaining stress tolerant plants, for example tolerant to salinity, to vectors comprising genetic information capable of conferring said tolerance to the plants, to muteins encoded by the said genetic information and to plants and plant materials obtainable by the said method.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Abiotic stress conditions, such as shortage or excess of solar energy, water and nutrients, salinity, high and low temperature and pollution (e.g., heavy metals), can have a major impact on plant growth and can significantly reduce the yield of, e.g., cultivars. In the state of the art it is known that, under conditions of abiotic stress, the growth of plant cells is inhibited by arresting the cell cycle in late G_1, before DNA synthesis, and/or at the G_2/M boundary; see reviews of Dudits, 1997, Plant Cell Division, Portland Press Research, Monograph, Francis, D., Dudits, D. and Inzé, D. eds, ch 2, pp 21, and Bergounioux, Protoplasma 142 (1988), 127-136.

The regulation of the cell cycle in plant cells is however poorly understood. WO 92/09685 generally describes a method for controlling plant cell growth comprising modulating the level of cell cycle control proteins in said plant cells. The method disclosed in WO92/09685 is described to be applicable for improvements of plant growth behavior in the presence of one or more environmental conditions. In particular, WO 92/09685 describes the presence of a $p34^{cdc2}$ protein in plants, a protein which is known to play a key role in the cell cycle of yeasts and vertebrates (see, e.g., the review by Lew and Kombluth, in Curr. Op. Cell Biol. 8 (1996), 795-804, herein incorporated by reference), wherein an indication is made that the amount of plant $p34^{cdc2}$ protein becomes limiting for cell division in plant tissue. However, no

clear indication was given as to the role of plant $p34^{cdc2}$ protein or other putative plant cell cycle control proteins in arresting the cell cycle under conditions of abiotic in particular salt stress nor related to the onset of cell cycle progress after the said cell cycle arrest.

Different approaches for the generation of stress tolerant plants have been described in the prior art. For example, WO 97/13843 describes the production of water stress or salt stress tolerant transgenic cereal plants by transforming the cereal plant cell or protoplast with a nucleic acid encoding a late embryogenesis abundant protein. Furthermore, the production of disease and stress tolerant plants by increasing the juvenility and antioxidant capacity was suggested; see Barna, Novenytermeles 44 (1995), 561-567. However, the above-described approaches have not been shown to be generally applicable and means that can be used to confer stress tolerance to plants without otherwise substantially affecting phenotype of the plant, e.g., growth characteristics, were hitherto not available.

Thus, the technical problem underlying the present invention is to provide means and methods for conferring or enhancing stress tolerance to plants which are particularly useful in agriculture.

The solution to the technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method for obtaining plants, tolerant to abiotic stress conditions, comprising introducing into a plant cell, plant tissue or plant a nucleic acid molecule and/or regulatory sequence, wherein the introduction of said nucleic acid molecule or regulatory sequence results in the presence of a Cyclin Dependent Kinase (CDK) protein that is not susceptible to inhibitory phosphorylation under abiotic stress conditions.

Control of CDK activity can be achieved by cyclin association and phosphorylation. The phosphorylation of CDK can either have an inhibitory effect or an activating effect on its activity depending on the position of the

phosphorylation site. $p34^{cdc2}$ is regulated by an activating phosphorylation during G2 at Thr 167 and by inhibitory phosphorylations at Thr-14 and/or Tyr-15 (Jacobs, Annu. Rev. Plant Physiol. Plant Mol Biol. 46 (1995), 317-339). The term "not susceptible to inhibitory phosphorylation under abiotic stress conditions" as used herein means that the CDK protein that is usually phosphorylated under stress conditions in the plant cell, is under-phosphorylated, i.e., non-phosphorylated at certain inhibitory phosphorylation sites which are otherwise phosphorylated under stress conditions. Thus, if, e.g., the CDK protein comprises four inhibitory phosphorylation sites which are usually phosphorylated in the plant cell under stress conditions, said CDK is present in a non-phosphorylated form in accordance with the present invention if at least one of said phosphorylation sites is unphosphorylated. The terms "not susceptible to inhibitory phosphorylation under abiotic stress conditions" and "non-phosphorylated form of a CDK protein" are used interchangeable herein.

The term "abiotic stress" as used herein refers to any adverse effect on metabolism, growth or viability of the cell, tissue, organ or whole plant which is produced by an non-living or non-biological (i.e., not biotic: insect, bacteria, fungal, virus) environmental stressor, e.g., environmental factors such as water (flooding, drought, dehydration), anaerobic (low level of oxygen, CO_2 etc.), osmotic (salt), temperature (hot/heat, cold, freezing, frost), nutrients/pollutants, or by a hormone, second messenger or other molecule which is related to or induced by said stressor.

The term "anaerobic stress" means any reduction in oxygen levels sufficient to produce a stress as hereinbefore defined, including hypoxia and anoxia.

The term "flooding stress" refers to any stress which is associated with or induced by prolonged or transient immersion of a plant, plant part, tissue or isolated cell in a liquid medium such as occurs during monsoon, wet season, flash flooding or excessive irrigation of plants, etc.

"Cold stress" and "heat stress" are stresses induced by temperatures which are respectively, below or above, the optimum range of growth temperatures for a particular plant species. Such optimum growth temperature ranges are readily determined or known to those skilled in the art.

"Dehydration stress" is any stress which is associated with or induced by the loss of water, reduced turgor or reduced water content of a cell, tissue, organ or whole plant.

"Drought stress" refers to any stress which is induced by or associated with the deprivation of water or reduced supply of water to a cell, tissue, organ or organism.

The terms "salinity-induced stress", "salt-stress" or similar term refer to any stress which is associated with or induced by a perturbation in the osmotic potential of the intracellular or extracellular environment of a cell.

The transgenic plant obtained in accordance with the method of the present invention, upon the presence of the nucleic acid molecule and/or regulatory sequence introduced into said plant, attains tolerance or improved tolerance against abiotic stress which the corresponding wild-type plant was susceptible to. The terms "tolerance" and "tolerant" cover the range of protection from a delay to complete inhibition of alteration in cellular metabolism, reduced cell growth and/or cell death caused by the abiotic stress defined hereinbefore. Preferably, the transgenic plant obtained in accordance with the method of the present invention is tolerant to abiotic stress in the sense that said plant is capable of growing substantially normal under environmental conditions where the corresponding wild-type plant shows reduced growth, metabolism, viability and/or male or female sterility.

Progression through the cell cycle is dependent on the activity of cyclin dependent kinases (CDKs) in all eukaryotes. In fission (Nurse and Bisset, Nature 292 (1981), 558-560) and budding yeast (Nasmyth, Curr. Opin. Cell. Biol. 5, (1993), 166-179) the CDC2 and respectively the CDC28 protein kinase are the central regulators of the cell cycle while in higher eukaryotes multiple CDKs with distinct roles are present (Mironov, V., De Veylder, V., Van Montagu, M. and Inzé, D. (1999), Molecular Control of the Cell Division Cycle in Higher Plants. The Plant Cell; and Pines, Sem. Cell. Biol. 5 (1994), 399-408). Dephosphorylation of CDC2 at tyrosine 15 in yeast (Gould, and Nurse, Nature 342 (1989), 39-45) and simultaneously at threonine 14 in animal cells by a CDC25 tyrosine phosphatase (Norbury, EMBO J. (1991), 3321-3329) is a prerequisite for cell cycle progression

into mitosis. Substitution of the Tyr15 residue to the non-phosphorylatable Phe15 results in fission yeast in small cells, the wee phenotype, as a consequence of premature mitotical entry (Russell and Nurse, Cell 49 (1987), 559-567). In *Arabidopsis*, the Thr14 and Tyr15 phosphorylation sites are conserved in the protein kinase CDC2aAt (Mironov (1999)). No phenotypic changes were however detected in transgenic *Arabidopsis* lines overexpressing a dominant negative mutant form of CDC2aAt with substituted Thr14 and Tyr15, except for a tendency to loose apical dominance (Hemerly, EMBO J. 14 (1995), 3925-3936).

In accordance with the present invention it was found that at the onset of the cell cycle arrest under abiotic stress conditions, the plant CDK protein, being functionally equivalent to the known CDC2a of *Arabidopsis thaliana*, was phosphorylated at a tyrosine and optionally also at a threonine residue, corresponding to the tyrosine of position 15 and the threonine of position 14 of said CDC2a respectively. Moreover, it was surprisingly found that the expression of non-phosphorylatable mutants of CDKs results in abiotic stress tolerant plants. The present invention is based on the finding that the transgenic plants overexpressing a mutant CDK, i.e., CDC2aAt with non-phosphorylatable Ala14 and Phe15 residues show increased tolerance to abiotic stress, in particular salt stress. Compared to wild type plants (WT) and transgenic *Arabidopsis* plants (YF2 and YF5, Example 2) ectopically expressing the wild type form of CDC2aAt (CDC2aWT), the YF lines displayed an enhanced shoot growth after cultivation in the presence of NaCl. Additionally the YF lines recovered faster upon release from salinity than the CDC2aWT and WT plants (Fig. 1). The results obtained in accordance with the present invention strongly suggest that the said phosphorylation in particular of plant CDK proteins appears to be one of the key events in abiotic stress-induced cell cycle arrest.

The terms "CDK" or "plant CDK" are meant to encompass all plant CDK proteins having a cell cycle regulatory function in plants or plant cells having the above-mentioned phosphorylatable tyrosine residue, and optionally in addition thereto, the said threonine residue. Examples of these CDK's are the members of the CDC2 family, as identified in *Arabidopsis thaliana*, such as CDC2a and CDC2b.

While the findings described above have been obtained with the CDK protein CDC2a of *Arabidopsis thaliana*, the present invention can be performed with any CDK protein that is functional in plants, i.e., plant CDK proteins, functionally equivalent to the known CDC2a of *Arabidopsis thaliana*. With "plant CDK protein, functionally equivalent to the known CDC2a of *Arabidopsis thaliana*" is meant each CDK protein having a similar regulatory function as CDC2a of *Arabidopsis thaliana* in plants or plant cells respectively, e.g., having the PSTAIRE conserved cyclin binding motif, and the above-mentioned phosphorylatable tyrosine and threonine residues. Intensive cloning efforts have identified a large number of CDK proteins in diverse plant species, among which at least five types can be distinguished on the basis of their sequences (for a compilation see Segers, In: Plant cell proliferation and its regulation in growth and development. Bryant JA, Chiatante D, editors. Chichester: John Wiley & Sons (1997), 1-19). In the model plant, *Arabidopsis thaliana,* two CDKs, each belonging to a different family, have been characterized. One such example is the *CDC2a*At gene, which contains the conserved PSTAIRE amino acid motif, and is constitutively expressed during the cell cycle at transcriptional and protein level. However, the associated kinase activity is maximal at the G1/S and G2/M transitions, suggesting a role at both checkpoints (Hemerly, Plant Cell 5 (1993) 1711-1723; Burssens, Plant Physiol. Biochem. 36 (1998), 9-19; Segers, Plant J. 10 (1996), 601-612). *CDC2b*At contains a PPTALRE motif and its mRNA levels are preferentially present during S and G2 phase (Segers, 1996 and references cited therein). The protein follows the transcriptional level but the CDC2b*At* kinase activity becomes only maximal during mitosis, implying a role during the M phase. Furthermore, CDKs or mutants thereof that can be employed in accordance with the present invention can be tested for their ability to confer abiotic stress tolerance to plants according to methods well-known in the art, see, e.g., Physical Stresses in Plants: Genes and Their Products for Tolerance. S. Grillo (Editor), A. Leone (Editor) (June 1996) Springer Verlag; ISBN: 3540613471; Handbook of Plant and Crop Stress. Mohammad Pessarakli (Editor), Marcel Dekker; ISBN: 0824789873; The Physiology of Plants Under Stress: Abiotic Factors. Erik T. Nilsen, David M. Orcutt (Contributor), Eric T. Nilsen. 2nd edition (October 1996), John Wiley &

Sons; ISBN: 0471031526; Drought, Salt, Cold, and Heat Stress: Molecular Responses in Higher Plants (Biotechnology Intelligence Unit). Kazuo Shinozaki (Editor), Kazuko Yamaguchi-Shinozaki (Editor) (1999). R G Landes Co; ISBN: 1570595631; Plants Under Stress: Biochemistry, Physiology and Ecology and Their Application to Plant Improvement (Society for Experimental Biology Seminar Serie). Hamlyn G. Jones, T.J. Flowers, M.B. Jones (Editor). (September 1989). Cambridge Univ. Pr. (Short); ISBN: 0521344239; Plant Adaptation to Environmental Stress. Leslie Fowden, Terry Mansfield, John Stoddart (Editor) (October 1993) Chapman & Hall; ISBN: 0412490005; or as described in the appended examples.

Determination of phosphorylation sites in CDKs corresponding to tyrosine at position 15 and the threonine of position 14 of CDC2a can be done, for example, by computer-assisted identification of such sites in the amino acid sequence of a given CDK using, e.g., BLAST2, which stands for Basic Local Alignment Search Tool (Altschul, 1997; Altschul, J. Mol. Evol. 36 (1993), 290-300; Altschul, J. Mol. Biol. 215 (1990), 403-410), which can be used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologues. Phosphorylating sites can also be determined using anti-phospho-tyrosine and anti-phospho-threonine antibodies as described by Zhang, Planta 200 (1996), 2-12.

The introduction of the nucleic acid molecule in the method of the present invention enhances the amount or results in de novo production of said non-phosphorylated form of CDK protein. For example, said nucleic acid molecule comprises a coding sequence of the mentioned protein or of a regulatory protein, e.g., a transcription factor, capable of inducing the expression of said CDK protein in its non-phosphorylated form or, e.g., of a CDK dephosphorylating enzyme or for example antisense to enzymes phosphorylating CDKs.

A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate

regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog.

The term "regulatory sequence" as used herein denotes a nucleic acid molecule increasing the expression of the said protein, e.g., the above-mentioned protein, due to its integration into the genome of a plant cell in close proximity to the gene, e.g., encoding inhibitors of phosphorylation. Such regulatory sequences comprise promoters, enhancers, inactivated silencer intron sequences, 3'UTR and/or 5'UTR coding regions, protein and/or RNA stabilizing elements or other gene expression control elements which are known to activate gene expression and/or increase the amount of the gene products.

The introduction of said nucleic acid molecule leads to de novo expression, or if the mentioned regulatory sequence is used to increase and/or induction of expression of said proteins, resulting in the end in an increased amount of a non-phosphorylated form of CDK protein in the cell. Thus, the present invention is aiming at providing de novo and/or increased expression of non-phosphorylated CDKs. In a preferred embodiment of the method of the present invention said CDK is a PSTAIRE type CDK, preferably said CDK is CDC2a.

As is demonstrated in the appended examples, it was found that in plants, at the onset of the cell cycle arrest under abiotic stress conditions, the endogenous Cyclin Dependent Kinases (CDK's) were phosphorylated at a tyrosine at position 15 and

optionally also at a threonine residue at position 14. In most plants for which CDK sequences have been identified, the positions of the said tyrosine and threonine residues are at positions 15 and 14, respectively; this is, e.g., the case for CDC2a of *Arabidopsis thaliana*. It is however possible that, e.g., during the course of evolution, in some plant CDK's the respective positions of these consensus Y-15 and T-14 have been shifted somewhat, i.e., as a result of one or more deletions or additions at the N-terminus of the protein.

The terms "tyrosine at position 15" and "threonine residue at position 14" as used herein, are therefor meant to encompass the positions 14 and 15 of the respective CDK, as well as such positional changes of the said tyrosine and threonine residues within the plant CDK protein, wherein the characteristic of these residues being phosphorylated at the onset of the stress-induced cell cycle arrest is retained. This means that the said positions as defined herein correspond to the tyrosine at position 15 and the threonine at position 14 of CDC2a of *Arabidopsis thaliana*, respectively.

Thus, in a preferred embodiment of the method of any one of the present invention, the CDK is free of phosphate at the tyrosine at a position that corresponds to position 15 in the amino acid sequence of CDC2a of *Arabidopsis thaliana*. Particularly preferred is that the CDK protein is free of phosphate groups at both the tyrosine and the threonine, corresponding to the tyrosine at position 15 and the threonine at position 14, respectively, in the amino acid sequence of CDC2a of *Arabidopsis thaliana*.

In one embodiment of the method of the present invention, said non-phosphorylated CDK protein is a non-phosphorylatable CDK mutein. A preferred embodiment of the present invention is by conferring to the plant the capacity to produce, under stress conditions, a CDK mutein, of which Y-15 is substituted to a non-phosphorylatable residue. When the plant is able to produce such a CDK mutein, said mutein will substantially not be sensitive for the phosphorylation system, triggering the stress-induced cell cycle arrest. In this way, the plant circumvents the downregulation of the cell cycle, being more tolerant to said stress conditions.

The term "CDK mutein", used herein, is defined as a CDK fragment or CDK protein

comprising at least one mutation, e.g., an amino acid substitution, deletion or addition. Additionally, phosphorylation of T-14 may play a potentiating or mediating role in the above-discussed downregulation mechanism. Therefore, in another preferred embodiment of the method according to the present invention, the CDK mutein also comprises a non-phosphorylatable amino acid residue at position 14.

Preferably, the said mutein is derived from endogenous CDK of the stress tolerant plant to be obtained. By starting from the endogenous CDK, the risk of malfunctioning muteins is minimized. However, in view of homology between different plant CDKs, it will be obvious to the skilled person that it is also possible to use CDK from another plant species. CDK, of, e.g., yeast or vertebrate origin may, dependent on the homology with the endogenous plant CDK, as well be suitable in the present invention; the suitability can easily be determined by the skilled person.

As a non-phosphorylatable amino acid residue substituting Y-15 (i.e., the tyrosine of the CDK, corresponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*), the CDK mutein preferably comprises a Y-15 -> F-15 mutation, F being phenylalanine. In all plants investigated so far, the expression of said mutein led to enhanced stress tolerance. Similarly, as a non-phosphorylatable amino acid residue substituting T-14 (i.e., the threonine of the CDK, corresponding to the threonine on position 14 of CDC2a of *Arabidopsis thaliana*), the CDK mutein preferably comprises a T-14 -> A-14 mutation, A being alanine. Expression of such a mutein led to improved stress tolerance.

As has been explained above, the method of the present invention can be performed in various ways. Thus, one could use, e.g., a plant cell that already comprises in its genome a nucleic acid molecule encoding a non-phosphorylatable form of CDK as described above, but does not express the same in an appropriate manner due to, e.g., a weak promoter. In such a case it would be sufficient to introduce into the plant cell a regulatory sequence such as a strong promoter in close proximity to the endogenous nucleic acid molecule encoding said non-phosphorylatable form of CDK so as to induc- expression of

the same. Usually, however, a wild-type plant cell will not have an endogenous gene encoding a non-phosphorylatable form of CDK. Therefore, in a preferred embodiment of the present invention said nucleic acid molecule to be introduced into the plant cell or plant tissue or plant encodes said non-phosphorylatable form of CDK.

Alternatively, the method of the present invention can be performed wherein said non-phosphorylated form of CDK is due to dephosphorylation and/or inhibition of phosphorylation of CDK.

As has been discussed above, it could surprisingly be shown that the downregulation of the cell division of plants, exposed to abiotic stress, was effectively counteracted by the presence of a CDK, in particular CDK, equivalent to CDC2a of *Arabidopsis thaliana*, being free of a phosphate at the Y-15 position. A preferred embodiment of the present invention therefore relates to conferring to the plant the capacity to provide, at the stress conditions, CDK protein, being functionally equivalent to CDC2a of *Arabidopsis thaliana*, a substantial portion thereof being free of phosphate at the tyrosine, corresponding to the tyrosine of position 15 of said CDC2a. A "substantial portion" in this respect is defined herein as the amount of CDK, being free of phosphate at the Y-15 position, that is sufficient to confer to the plant improved growth during abiotic stress conditions. The person skilled in the art will understand that not all of the corresponding CDK present in the plant or plant cell has to be phosphate free at the Y-15 position to improve said stress tolerance. This may, e.g., be accomplished by conferring to the plant the capacity of preventing phosphorylation of the said tyrosine, or of activating the dephosphorylation mechanism for the said tyrosine. As this counteraction may further be improved upon T-14 being additionally free of phosphate, the CDK protein is preferably free of phosphate groups at both the tyrosine and the threonine, corresponding to the tyrosine on position 15 and the threonine on position 14 of said CDC2a, respectively.

An attractive way to obtain stress tolerant plants according to the present invention is therefore by conferring to the plant the capacity to provide under stress conditions, CDC25 or a functional analogue thereof, capable of dephosphorylating at least the

tyrosine at position 15 of the endogenous CDK of the said plant. The dephosphorylating activity of CDC25 is described in Lew and Kornbluth, *supra*. By enabling the plant to produce, at the stress conditions, functional CDC25 protein, i.e., capable of dephosphorylating the above-mentioned tyrosine, and optionally also the adjacent threonine of the endogenous CDK, the phosphorylation of CDK as a result of the stress conditions is effectively counteracted.

As well in mammals as in yeast the function of the WEE1 protein kinase is antagonistic to CDC25, acting as a mitotic inhibitor by phosphorylation of CDC2 on Tyr15 (Igarashi, Nature 353 (1991), 80-3; Russell and Nurse, Cell 49 (1987), 559-567; Labib and Nurse, Current Biology, 3 (1993), 164-166). A Wee 1 plant homologue from maize, ZmWee1 has recently been identified (Sun, Proc. Natl. Acad. Sci. USA 96 (1999), 4180-4185). In fission yeast MIK1 acts cooperatively with the WEE1 protein kinase in the inhibitory Tyr15 phosphorylation of CDC2 (Lundgren, Cell 64 (1991), 1111-1122). In *Xenopus* a MYT1 kinase has been identified that phosphorylates CDC2 at both Tyr15 and Thr 14 to keep the CDC2 complex in a mitotic inactive state (Mueller, Science 270 (1998), 86-89).

Thus, another attractive route to obtain stress tolerant plants according to the present invention is by conferring to the plant the capacity to inhibit, under stress conditions, the expression or activity of at least Wee-kinase, MIK1 or MYT or a functional equivalent thereof, thereby inhibiting or reducing the endogenous phosphorylation of CDK of the said plant at least the tyrosine at position 15. Wee-kinase is reviewed in, e.g., Lew and Kornbluth, *supra*. This kinase phosphorylates the above-discussed Y-15 of CDK and may also be responsible for the phosphorylation of the T-14. With "functional equivalent of Wee-kinase" is meant any endogenous kinase of the plant having the function of known Wee-kinase in phosphorylating the respective tyrosine residue and optionally the threonine residue of the endogenous plant CDK. The recently identified Myt1 kinase (Mueller, Science 270 (1995), pp 86) may therefore be regarded as such a functional equivalent. By inhibiting the expression of the Wee-kinase under abiotic stress conditions, the phosphorylation of CDK will be inhibited, reducing the downregulation of cell division (mitotic activity) and growth, thus

obtaining stress tolerance.

Thus, engineering of transgenic plants in accordance with the present invention comprises the use of the animal or yeast *CDC25*, *WEE1*, *MYT1* or *MIK1* genes or more preferably their plant homologues such as Wee1 form maize; see Sun, supra.

Strategies include overexpressing *CDC25* homologue by use of a regulatory sequence described herein and knock out of protein kinases (WEE, MYT and MIK) by, e.g., RNA antisense constructs, t-DNA insertion, cosuppression, dominant negative mutants, homologous recombination technology, etc. described in more detail below.

As will be appreciated by the person skilled in the art, the expression of the above-described phosphatases or inhibition of said protein kinases can be achieved in different ways. For example, the expression of the plant homologue of CDC25 can be induced by introduction of a regulatory sequence as defined above so as to induce the expression of the endogenous phosphatase gene. Furthermore, it is possible to inhibit phosphorylation by the mentioned kinases through inhibition of gene expression of the said protein kinases or inactivation of their gene products. In a preferred method of the present invention, said nucleic acid molecule introduced into the plant cell, plant tissue or plant encodes said CDC25 or its functional analog. In humans, three phosphatases have been identified in : *CDC25a*, *b*, and *c*. CDC25a plays a role at the G1/S transition while CDC25b and c are considered to be functional at the G2/M transition (Sahdu, Proc. Natl. Acad. Sci. USA 87 (1990), 5139-5143; Galaktiniov, Cell 67 (1991), 1181-1194; Nagata, New Biol. 10 (1991), 959-968; Jinno, EMBO J. 13 (1994), 1549-1556). In fission yeast (*S.pombe*) one *CDC25* phosphatase has been isolated (Gould and Nurse, Nature 342 (1989), 39-45; Labib and Nurse (1993)). Although no CDC25 cognate has yet been isolated in plants, considering the evolutionary character of the cell cycle, similar phosphatases might however exist in plants (cfr. Tyr15 phosphorylation of CDC2 like protein kinases is suggested by the work of Zhang, Planta 200 (1996), 2-12; Schuppler, Plant Physiol. 117

(1998), 667-678, and demonstrated by the analysis of the YF plants upon salinity stress as presented in the examples). The phenotypic analysis of transgenic plants that overexpress the fission yeast *CDC25* by McKibbin, Plant Mol. Biol. 36 (1996), 601-612 confirms this hypothesis. These plants produced more lateral roots, implying that pericycle cells, from which lateral roots are initiated, bypass a checkpoint, which is relieved by the action of the fission yeast CDC25.

In a further preferred embodiment, said nucleic acid molecule to be introduced into the plant cell or plant tissue or plant encodes an antisense RNA of said WEE kinase, MYT, MIK or functional analogue or equivalent thereof.

In case the above-described proteins or at least one of them are to be expressed de novo, it is preferred to employ in the method of the present invention genes encoding such proteins, e.g., CDK muteins, wherein said gene is expressible in plant cells. Thus, in another embodiment the method of the present invention said nucleic acid molecule is operatively linked to regulatory sequences allowing the expression of the nucleic acid molecule in the plant. Said regulatory sequences comprise a promoter, enhancer, silencer, intron sequences, 3'UTR and/or 5'UTR regions, protein and/or RNA stabilizing elements. Preferably, said regulatory sequence is a chimeric, tissue specific, constitutive or inducible promotor. The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used. Furthermore, the nucleic acid molecule to be used in accordance with the present invention can be operably linked to poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV) and from the Nopaline Synthase promoter. Additional regulatory elements may include transcriptional as well as translational enhancers. A plant translational enhancer often used is the tobaccos mosaic virus (TMV) omega sequences, the inclusion of

an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676).

Any promoter that functions in the target cells can be used. The use of the CaMV35S promoter, per se known to the skilled person, resulted in plants with improved tolerance to salt stress (i.e., to a salt concentration in the growth medium of, e.g., 1 w/v % NaCl). It is preferable to use a promoter, that can be induced upon the abiotic stress conditions. Such promoters can be taken for example from stress-related genes which are regulated directly or indirectly by an environmental, i.e., preferably abiotic, stress in a plant cell, including genes for which expression is increased, reduced or otherwise altered. These stress-related genes comprise genes the expression of which is either induced or repressed by anaerobic stress, flooding stress, cold stress, dehydration stress, drought stress, heat stress or salinity, amongst others. For example, the stress-related gene may encode an ANP selected from the group consisting of sucrose synthase, phosphoglucomutase, phosphoglucose isomerase, fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase, pyruvate decarboxylase, alcohol dehydrogenase and alanine amino transferase, amongst others. Such promoters are known in the art (see also, e.g., Lang, and Palva, Plant Mol. Biol. 20 (1992), 951 and Table 1 below).

Table 1

Name	**Stress**	Reference
P5CS (delta(1)-pyrroline-5-carboxylate synthase)	salt, water	Zhang, Plant Science 129 (1997), 81-89
cor15a	cold	Hajela, Plant Physiol. 93 (1990), 1246-1252
cor15b	cold	Wilhelm, Plant Mol Biol. 23 (1993), 1073-7
cor15a (-305 to +78 nt)	cold, drought	Baker, Plant Mol Biol. 24

Name	Stress	Reference
		(1994), 701-13
rd29	salt, drought, cold	Kasuga, Nature Biotechnology 18 (1999), 287-291
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros, Plant Mol. Biol. 19(4) (1992), 665-75; Marrs, Dev Genet. 14(1) (1993), 27-41; Schoffl, Mol. Gen. Genet. 217(2-3) (1989), 246-53
smHSP (small heat shock proteins)	heat	Waters, J. Experimental Botany 47, 296 (1996), 325-338,
wcs120	cold	Ouellet, FEBS Lett. 423 (1998), 324-328
ci7	cold	Kirch, Plant Mol. Biol. 33 (1997), 897-909
Adh	cold, drought, hypoxia	Dolferus, Plant Physiol, 105(4) (1994), 1075-87
pwsi18	water: salt and drought	Joshee, Plant Cell Physiol. 39 (1998), 64-72
ci21A	cold	Schneider, Plant Physiol. 113 (1997), 335-45
Trg-31	drought	Chaudhary, Plant Mol. Biol. 30 (1996), 1247-57
osmotin	osmotic	Raghothama, Plant Mol. Biol. 23 (1993), 1117-28

In a particularly preferred embodiment of the method of the present invention said inducible promoter is inducible by abiotic stress, preferably, said abiotic stress is osmotic stress, preferably caused by salt.

Preferably, the above-described nucleic acid molecules are comprised in an expression vector. An "expression vector" is a construct that can be used to

transform a selected host cell and provides for expression of a coding sequence or antisense in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Advantageously, the above-described vectors of the invention comprise a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for *dhfr*, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); *npt*, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and *hygro*, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable markers are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention.

The present invention also relates to vectors, particularly plasmids, cosmids,

viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain at least one nucleic acid molecules and/or regulatory sequences according to the invention. In particular, the present invention relates to a vector, at least comprising a stress-inducible promoter, preferably, a salt stress-inducible promoter, functional in plant cells, operably linked to a DNA sequence, coding for a mutated *cdc2a* gene of *Arabidopsis thaliana* or functionally equivalent gene of another species, preferably a plant species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising, in the CDK mutein, a non-phosphorylatable amino acid residue at the position, corresponding to the tyrosine on position 15 of CDC2a. Preferably, the mutein also comprises a non-phosphorylatable amino acid residue at the position of the mutein, corresponding to the threonine on position 14 of said CDC2a. In order to minimize the possibility of malfunctioning, it is preferred that a plant CDK gene is used. Being transformed with a vector of this type, plant cells are capable of producing, at abiotic stress conditions, CDK muteins that are not susceptible to the above-discussed regulatory phosphorylation events, therefor leading to stress tolerant plants or plant cells. In a further embodiment, the vector comprises a promoter as defined above, functional in plant cells, operably linked to a DNA sequence, coding for CDC25 or a functional analogue thereof, capable of dephosphorylating at least the tyrosine of at least one plant CDK, corresponding with the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*. Such a vector can be used to transform plants in order to, as is discussed above, express CDC25 in plants, resulting in dephosphorylation of Y-15 and optionally the T-14 of the endogenous plant CDK, leading to improved stress tolerance.

Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Plasmids and vectors to be preferably employed in accordance with the present invention include those well known in the art. Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for

delivery to target cells. The present invention furthermore relates to host cells comprising a vector as described above wherein the nucleic acid molecule is foreign to the host cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally.

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae.

In a preferred method for obtaining stress tolerant plants according to the present invention, the said capacity is conferred to one or more cells of said plant by a) transforming one or more plant cells with a vector, at least comprising, under the control of a promoter functional in the said plant cells, a DNA sequence, coding for a mutated *cdk* gene of *Arabidopsis thaliana* or functional equivalent gene of another species, the gene product thereof being a CDK mutein functional in the said plant cells and comprising a non-phosphorylatable amino acid residue at the position of the CDK mutein, corresponding to the tyrosine on position 15 of CDC2a of *Arabidopsis thaliana*, preferably, the mutein also comprises a non-phosphorylatable amino acid residue at position 14 of the CDK mutein b) by regenerating a plant from one or more of the transformed plant cells, e.g., by the *Agrobacterium tumefaciens* transformation

system. However, other transformation methods known in the field may be used. With "mutein, functional in plant cells", muteins are meant, which, when expressed in the said plant cells, lead to improved stress tolerance of the said cells.

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e., the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example co-transformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of *Agrobacterium tumefaciens* and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic Acid Res.

12 (1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of *Agrobacterium tumefaciens* is preferred in the method of the invention, other Agrobacterium strains, such as *Agrobacterium rhizogenes*, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou, Trends in Plant Science 1 (1996). 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995). The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, Agrobacterium mediated transformation etc.

The term "transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. The resulting transformed plant cell or plant tissue can then be used to regenerate a transformed plant in a manner known by a skilled person.

In general, the plants which can be modified according to the invention and which show over- and/or de novo expression of a non-phosphorylated form of a CDK

protein can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as a crop plant, root plant, oil producing plant, wood producing plant, agricultured bioticultured plant, fruit-producing plant, fodder or forage legume, companion plant, or horticultured plant e.g., such a plant is wheat, barley, maize, rice, carrot, sugar beet, chicory, cotton, sunflower, tomato, cassava, grapes, soybean, sugar cane, flax, oilseed rape, tea, canola, onion, asparagus, carrot, celery, cabbage, lentil, broccoli, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, rye, sorghum, oats, tobacco, pepper, grape or potato. Additional species are not excluded. Crops grown on cultivated lands in arid or semi-arid areas in which irrigation with ground water is needed may advantageously benefit from the invention.

Thus, the present invention relates also to transgenic plant cells which contain a nucleic acid molecule or regulatory sequence as defined above or vector according to the invention wherein the nucleic acid molecule or regulatory sequence is foreign to the transgenic plant cell. For the meaning of the term "foreign"; see supra.

In one aspect the present invention relates to a transgenic plant cell comprising stably integrated into the genome a nucleic acid molecule, regulatory sequence, or a vector in accordance with the present invention or obtainable according to the method of the invention wherein the expression of the nucleic acid molecule or conferred by the regulatory sequence results in an increased or de novo expression of a non-phosphorylated form of CDK or of a dephosphorylating enzyme described above in transgenic plants compared to wild-type plants. Alternatively, a plant cell having a nucleic acid molecule encoding a CDK mutein or corresponding dephosphorylating enzyme present in its genome can be used and modified such that said plant cell expresses the endogenous gene corresponding to this nucleic acid molecule under the control of regulatory sequences described above such as heterologous promoter and/or enhancer elements. The introduction of the heterologous promoter and mentioned elements which do not naturally control

the expression of a nucleic acid molecule encoding, e.g., a CDK mutein using, e.g., gene targeting vectors can be done according to standard methods, see supra and, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular Biology* 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115). Suitable promoters and other regulatory elements such as enhancers include those mentioned herein before.

In another aspect, the present invention relates to a transgenic plant cell which contains stably integrated into the genome a nucleic acid molecule, regulatory sequence or vector described above or obtainable according to the method of the invention, wherein the presence, transcription and/or expression of the nucleic acid molecule, regulatory sequences or part thereof leads to reduction of the synthesis or the activity of proteins phosphorylating CDKs under abiotic stress conditions in transgenic plants compared to wild type plants. Preferably, said reduction is achieved by an antisense, sense, ribozyme, co-suppression, in vivo mutagenesis, antibody expression and/or dominant mutant effect. Therefore, the use of nucleic acid molecules encoding an antisense RNA which is complementary to transcripts of an enzyme phosphorylating CDK in a plant is also the subject matter of the present invention. Thereby, complementarity does not signify that the encoded RNA has to be 100% complementary. A low degree of complementarity is sufficient, as long as it is high enough in order to inhibit the expression of a protein phosphorylating CDK upon expression in plant cells. The transcribed RNA is preferably at least 90% and most preferably at least 95% complementary to the transcript of the nucleic acid molecule encoding such a phosphorylating enzyme. In order to cause an antisense-effect during the transcription in plant cells such DNA molecules have a length of at least 15 bp, preferably a length of more than 100 bp and most preferably a length or more than 500 bp, however, usually less than 5000 bp, preferably shorter than 2500 bp. Also DNA molecules can be employed which, during expression in plant cells, lead to the synthesis of an RNA which in the plant cells due to a co-suppression-effect reduces the expression of the nucleic acid molecules encoding the described

phosphorylating protein. The principle of the co-suppression as well as the production of corresponding DNA sequences is precisely described, for example, in WO 90/12084. Such DNA molecules preferably encode an RNA having a high degree of homology to transcripts of the genes encoding phosphorylating enzymes. It is, however, not absolutely necessary that the coding RNA is translatable into a protein. The principle of co-suppression effect is known to the person skilled in the art and is, for example, described in Jorgensen, Trends Biotechnol. 8 (1990), 340-344; Niebel, Curr. Top. Microbiol. Immunol. 197 (1995), 91-103; Flavell, Curr. Top. Microbiol. Immunol. 197 (1995), 43-36; Palaqui and Vaucheret, Plant. Mol. Biol. 29 (1995). 149-159; Vaucheret, Mol. Gen. Genet. 248 (1995), 311-317; de Borne, Mol. Gen Genet. 243 (1994), 613-621 and in other sources.

Likewise, DNA molecules encoding an RNA molecule with ribozyme activity which specifically cleaves transcripts of a gene encoding the dephosphorylating enzyme can be used. Ribozymes are catalytically active RNA molecules capable of cleaving RNA molecules and specific target sequences. By means of recombinant DNA techniques it is possible to alter the specificity of ribozymes. There are various classes of ribozymes. For practical applications aiming at the specific cleavage of the transcript of a certain gene, use is preferably made of representatives of two different groups of ribozymes. The first group is made up of ribozymes which belong to the group I intron ribozyme type. The second group consists of ribozymes which as a characteristic structural feature exhibit the so-called "hammerhead" motif. The specific recognition of the target RNA molecule may be modified by altering the sequences flanking this motif. By base pairing with sequences in the target molecule these sequences determine the position at which the catalytic reaction and therefore the cleavage of the target molecule takes place. Since the sequence requirements for an efficient cleavage are low, it is in principle possible to develop specific ribozymes for practically each desired RNA molecule.

In order to produce DNA molecules encoding a ribozyme which specifically cleaves transcripts of a gene encoding a kinase for CDK, for example a DNA sequence

encoding a catalytic domain of a ribozyme is bilaterally linked with DNA sequences which are homologous to sequences encoding the target protein. Sequences encoding the catalytic domain may for example be the catalytic domain of the satellite DNA of the SCMo virus (Davies, Virology 177 (1990), 216-224 and Steinecke, EMBO J. 11 (1992), 1525-1530) or that of the satellite DNA of the TobR virus (Haseloff and Gerlach, Nature 334 (1988), 585-591). The DNA sequences flanking the catalytic domain are preferably derived from the above-described DNA molecules of the invention. The expression of ribozymes in order to decrease the activity in certain proteins in cells is also known to the person skilled in the art and is, for example, described in EP-B1 0 321 201. The expression of ribozymes in plant cells was, for example, described, in Feyter et al. (Mol. Gen. Genet. 250 (1996), 329-338).

Furthermore, the kinase activity of enzymes capable of phosphorylating CDK in the plant cells of the invention can also be decreased by the so-called "in vivo mutagenesis", for which a hybrid RNA-DNA oligonucleotide ("chimeroplast") is introduced into cells by transformation of cells TIBTECH 15 (1997), 441-447; WO95/15972; Kren, Hepatology 25 (1997), 1462-1468; Cole-Strauss, Science 273 (1996), 1386-1389). Part of the DNA component of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous enzyme capable of phosphorylating CDK, in comparison to the said nucleic acid sequence protease it displays, however, a mutation or contains a heterologous region which is surrounded by the homologous regions. By means of base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule followed by a homologous recombination the mutation contained in the DNA component of the RNA-DNA oligonucleotide or the heterologous region can be transferred to the genome of a plant cell. This results in a decrease of the activity.

Furthermore, nucleic acid molecules encoding antibodies specifically recognizing an enzyme capable of phosphorylating a CDK in a plant or parts, i.e., specific fragments or epitopes, of such a protein can be used for inhibiting the activity of the protein in plants. These antibodies can be monoclonal antibodies, polyclonal antibodies or

synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Expression of antibodies or antibody-like molecules in plants can be achieved by methods well known in the art, for example, full-size antibodies (Düring, Plant. Mol. Biol. 15 (1990), 281-293; Hiatt, Nature 342 (1989), 469-470; Voss, Mol. Breeding 1 (1995), 39-50), Fab-fragments (De Neve, Transgenic Res. 2 (1993), 227-237), scFvs (Owen, Bio/Technology 10 (1992), 790-794; Zimmermann, Mol. Breeding 4 (1998), 369-379; Tavladoraki, Nature 366 (1993), 469-472) and dAbs (Benvenuto, Plant Mol. Biol. 17 (1991), 865-874) have been successfully expressed in Tobacco, Potato (Schouten, FEBS Lett. 415 (1997), 235-241) or *Arabidopsis*, reaching expression levels as high as 6.8% of the total protein (Fiedler, Immunotechnology 3 (1997), 205-216).

In addition, nucleic acid molecules encoding mutant forms of a protein capable of phosphorylating a CDK in a plant protease can be used to interfere with the activity of the wild type protein. Such mutant forms preferably have lost their biological activity, e.g., kinase activity and may be derived from the corresponding wild-type protein by way of amino acid deletion(s), substitution(s), and/or additions in the amino acid sequence of the protein. Mutant forms such proteins also encompass hyper-active mutant forms of such proteins which display, e.g., an increased substrate affinity and/or higher substrate turnover of the same. Furthermore, such hyper-active forms may be more stable in the cell due to the incorporation of amino acids that stabilize proteins in the cellular environment. These mutant forms may be naturally occurring or genetically engineered mutants, see also supra.

The nucleic acid and amino acid sequences for proteins capable of phosphorylating CDK in a plant can be arrived, for example, from the above-

described Wee-kinase MIK or MYT proteins. Furthermore, it is immediately evident to the person skilled in the art that the above-described antisense, ribozyme, co-suppression, in-vivo mutagenesis, antibody expression and dominant mutant effects can also be used for the reduction of the expression of genes that encode a regulatory protein such as transcription factors that control the expression of enzymes capable of phosphorylating CDK in plant cells. Likewise the described methods can be used, for example, to knock-out the activity of regulatory proteins that, for example, are necessary for CDK phosphorylating enzymes to become active. Furthermore, the above-described methods can be used to knock-out the expression or activity of the endogenous wild-type forms of CDKs in plant cells. This would have the advantage that a CDK mutein in the plant cell does not have to compete with the wild-type form and that therefore, lower levels of CDK muteins may be sufficient so as to achieve the desired phenotype.

It is also evident from the disclosure of the present invention, that any combination of the above-identified strategies can be used for the generation of transgenic plants, which due to the presence of non-phosphorylated form of CDK display a novel or enhanced abiotic stress tolerance. Such combinations can be made, e.g., by (co-)transformation of corresponding nucleic acid molecules into the plant cell, plant tissue or plant, or may be achieved by crossing transgenic plants that have been generated by different embodiments of the method of the present invention. Likewise, the plants obtainable by the method of the present invention can be crossed with other transgenic plants so as to achieve a combination of abiotic stress tolerance and another genetically engineered trait, see also infra.

In addition, the present invention also relates to transgenic plants and plant tissue comprising transgenic plant cells according to the invention. Said transgenic plant cell comprises at least one nucleic acid molecule or regulatory sequence as defined above or obtainable by the method of the present invention. Furthermore, the present invention relates to transgenic plants and plant tissue obtainable by

the method of the present invention. As mentioned above, said transgenic plants may display various idiotypic modifications due to their abiotic stress tolerance, preferably display accelerated and/or enhanced plant growth, root growth and/or yield compared to the corresponding wild type plant.

As described before, the plant cells, plant tissue, in particular, transgenic plants of the invention display a certain degree of (higher) abiotic stress resistance compared to the corresponding wild-type plants. For the meaning of "abiotic stress"; see supra. In a preferred embodiment of the present invention, the transgenic plant displays increased tolerance to osmotic stress, preferably to salt stress. An increase in tolerance to salt stress is understood to refer to the capability of the transgenic plant to grow on a medium such as soil, comprising a higher content of salt in the order of at least about 10% compared to a medium the corresponding non-transformed wild-type plants is capable to grow on, which already provides for beneficial effects on the vitality of the plant such as, e.g., improved growth. Advantageously, the transgenic plant of the invention is capable of growing on a medium or soil comprising at least about 50%, preferably more than about 75%, particularly preferred at least about more than 100% and still more preferable more than about 200% salt than medium or soil the corresponding wild-type plant is capable of growing on.

In a particular preferred embodiment of the present invention, the above-described transgenic plants are capable of growing on medium or soil containing 40, more preferably 100, still more preferably 200, and even more advantageously 300 mM salt. Said salt can be for example, water soluble inorganic salts such as sodium sulfate, magnesium sulfate, calcium sulfate, sodium chloride, magnesium chloride, calcium chloride, potassium chloride etc., salts of agricultural fertilizers and salts associated with alkaline or acid soil conditions. Preferably, said salt is NaCl.

Any transformed plant obtained according to the invention can be used in a conventional breeding scheme or in *in vitro* plant propagation to produce more

transformed plants with the same characteristics and/or can be used to introduce the same characteristic in other varieties of the same or related species.

Furthermore, the characteristic of the transgenic plants of the present invention to maintain rapid/high growth rates under stress conditions can be combined with various approaches to confer biotic or abiotic stress tolerance with the use of other stress tolerance genes. Some examples of such stress tolerant genes are provided in Table 2 and see generally Holmberg and Bulow, Trends Plant Sci. 3, 61-66 (1998). Most prior art approaches which include the introduction of various stress tolerance genes have the drawback that they result in reduced growth (compared to non-transgenic controls) under normal, non-stressed conditions, namely stress tolerance comes at the expense of growth and productivity. This correlation between constitutive expression of stress-responsive genes and reduced growth rate under normal growth conditions indicates the presence of cross talk mechanisms between stress response control and growth control. Therefore, making cell division (and growth) insensitive to stress control by preventing or removing inhibitory phosphorylation of CDK protein will also result in faster growth of plants that constitutively express stress-tolerance mechanisms under non-stressed conditions.

Furthermore, the characteristic of the transgenic plants of the present invention to display abiotic stress tolerance can be combined with various approaches to confer to plants other stress tolerance genes, e.g., osmotic protectants such as mannitol, proline; glycine-betaine, water-channeling proteins, etc. Thus, the approach of the present invention to confer abiotic stress tolerance to plants can be combined with prior art approaches which include introduction of various stress tolerance genes; see, e.g., Table 2.

Table 2

Stress tolerance gene	**Reference**
pyrroline-5-carboxylate synthetase	Kishor, Plant Physiol. 108 (1995), 1387-1394.
mannitol	Tarczynski, Science 259 (1993), 508-510

Stress tolerance gene	Reference
	Holmstrom, Nature (1996), 683-684
fructan	Pilon-Smith, Plant Physiol. 107(1995), 125-130
COR	Jaglo-Ottensen, Science 280 (1998), 104-106
codA	Hayashi, Plant J. 12 (1997), 133-142
w-3 fatty acid desaturase	Kodama, Plant Physiol. 105 (1994), 601-605
Delta9 desaturase	Ishizaki-Nishizawa, Nat. Biotechnol. 14 (1996), 1003 –1006
rd29A, rd17, cor6.6, cor15a, erd10, kin1	Liu-Q, Plant Cell. Aug. 10 (8) (1998), 1391-1406
MYB2	WO99/16878
CaN-calcineurin	WO99/05902
water channel proteins	WO98/17803
late embryogenesis abundant protein HVA1	WO97/13843
mannitol	US 5,780,709

Thus, due to the findings of the present invention, it is now also possible to produce transgenic plants which have the ability to grow under abiotic stress conditions and display further new phenotype characteristics compared to naturally occurring wild-type plants, for example, due to the presence of another transgene. Hence, the above-described nucleic acid molecules and regulatory sequences can be used in combination with other transgenes that confer another phenotype to the plant. Likewise, it is possible to first confer abiotic stress tolerance to a plant in accordance with the method of the invention and to then in an additional step transform such plant in accordance thereof with a further nucleic acid molecule, the presence of which results in another new phenotype characteristic of said plant. Irrespective of the actual performance of

transformation, the result of the present invention displays at least two new properties compared to a naturally occurring wild-type plant, that is increased tolerance to abiotic stress, in particular osmotic stress preferably to high salinity and; a phenotype that is due to the presence of a further nucleic acid molecule in said plants. For example, said phenotype is conferred by the (over)expression of homologous or heterologous genes or suppression of endogenous genes of the plant or their gene products.

Some examples for the (over)expression of homologous or heterologous genes and antisense inhibition and co-suppression aiming at manipulating certain metabolic pathways in transgenic plants are reviews in Herbers (TIBTECH 14 (1996), 198-205): Ribozymes of different kinds which are capable of specifically cleaving the (pre)-mRNA of a target gene are described in, e.g., EP-B1 0 291 533, EP-A10 321 201 and EP-A2 0 360 257. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. An example for ribozyme mediated virus resistance is described in Feyter (Mol. Gen. Genet. 250 (1996), 329-228). Thus, it is immediately evident to the person skilled in the art that the method of the present invention can be employed to produce transgenic stress tolerant plant with any further desired trait (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397) comprising (i) herbicide tolerance (DE-A-3701623; Stalker, Science 242 (1988), 419), (ii) insect resistance (Vaek, Plant Cell 5 (1987), 159-169), (iii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology & Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996) 56 suppl.), (vi) ozone resistance (Van Camp, Biotech. 12 (1994), 165-168), (v) improving the preserving of fruits (Oeller, Science 254 (1991), 437.439), (vi) improvement of starch composition and/or production (Stark, Science 242 (1992), 419; Visser, Mol. Gen. Genet. 225 (1991), 289-296), (vii) altering lipid composition (Voelker, Science 257 (1992), 72-74), (viii) production of (bio)polymers (Poirer, Science 256 (1992), 520-523), (ix) alteration of the flower color, e.g., by manipulating the anthocyanin and flavonoid biosynthetic pathway (Meyer, Nature 330 (1987), 667-678, WO 90/ 12084), (x)

resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141), (xi) alteration of alkaloid and/or cardiac glycoside composition, (xii) inducing maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO 93/25695); (xiii) higher longevity of the inflorescences/flowers, and (xvi) stress resistance; see references supra, e.g., those mentioned in Table 2.

Thus, the present invention relates to any plant cell, plant tissue, or plant which due to genetic engineering displays abiotic stress tolerance obtainable in accordance with the method of the present invention and comprising a further nucleic acid molecule conferring a novel phenotype to the plant such as one of those described above.

As mentioned before, the combination of the approaches can be done by crossing plants displaying the individual phenotypes referred to above. Such plants are also part of the invention. Seeds obtained from the transformed plants genetically also contain the same characteristic and are part of the invention. As mentioned before, the present invention is in principle applicable to any plant and crop that can be transformed with any of the transformation method known to those skilled in the art and includes those mentioned hereinbefore for instance corn, wheat, barley, rice, oilseed crops, cotton, tree species, sugar beet, cassava, tomato, potato, numerous other vegetables, fruits.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells according to the invention. Harvestable parts can be in principle any useful parts of a plant, for example, flowers, pollen, seedlings, tubers, leaves, stems, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

It is to be understood that the skilled person, aware of the above teaching, will be

able to apply numerous techniques to confer to a plant the capacity to counteract the stress-induced downregulation of cell cycle progress as is discussed above. Instead of or in addition to transforming plant cells with a gene coding for a CDK mutein, it is possible to overexpress in the plant cells CDC25 or functional analogue thereof by transforming the said cells with a functional *cdc25* gene under the control of a suitable promoter, e.g., the CaMV35S promoter, or, e.g., to transform the plants with nucleic acids coding for anti-sense RNA, capable to basepair with, and leading to cleavage of, the mRNA coding for any protein that is desired to be knocked out, like Wee-kinase-mRNA.

Thus, the present invention generally relates to the use of the above described nucleic acid molecules, regulatory sequences, and vectors for conferring abiotic stress tolerance to a plant and/or as a selectable marker for plants.

The above described nucleic acid molecules, regulatory sequences and vectors in accordance with the method of the invention for conferring abiotic stress tolerance can be used as selectable markers in plants according to other systems which for example employ (over)expression of enzymes or muteins thereof capable of conferring tolerance (i.e., resistance) to plant cell killing effects of, e.g., herbicides. An example for such a system is the overexpression of the enzyme 5-enolpyruvlyshikimate-3-phosphate (EPSP) synthase that confers tolerance to the herbicide glyphosphate. In a similar way, the nucleic acid molecules, regulatory sequences and vectors described above can be used for conferring tolerance against abiotic stress, in particular salt stress as shown in the appended examples. For example, transgenic plants obtained in accordance with the method of the present invention can be easily selected for in the green house on soil, which contains, for example 40 to 300 mM salt, e.g., NaCl.

As has been discussed hereinbefore, in investigating the behavior of plant cells and plants under conditions of abiotic stress, it could be shown in accordance with the present invention that stress-dependent downregulation of the cell division (cell cycle) is mediated by endogenous cellular components. Said components may comprise cell cycle regulatory proteins that may undergo stress induced alterations,

thereby being activated or deactivated. Moreover, it could surprisingly be shown that one could confer to the plant the capacity to counteract or even avoid the downregulation of the cell division under conditions of abiotic stress, in particular osmotic stress due to, e.g., high salinity of the soil, thus enabling the plant to be tolerant to the said stress conditions, by, e.g., altering, or inhibiting, or competing with, or circumventing the regulatory actions of, the above-mentioned endogenous cellular components. Thus, in a still further embodiment, the present invention relates to the use of a nucleic acid molecule or regulatory sequence capable of counteracting stress-induced down-regulation of cell division for the production for osmotic stress tolerant plants.

Furthermore, the present invention relates to the use of a plant obtainable by the method of the invention or a plant as described hereinbefore for culturing on soil with 40 mM to 300 mM salt content.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The figures show:

Fig. 1: Recovery from salt stress of wild type (WT), CDC2aWT, YF2 and YF5

lines.

(A) Control plants after seven days transfer to K1 medium

(B) Salt stressed plants after seven days transfer to K1 medium containing 1%NaCl

(C) Control plants after fourteen days transfer to K1 medium

(D) Salt stressed plants after seven days recovery from a 1% containing K1 medium

Fig. 2: Differential growth upon salt stress of the WT, CDC2aWT, YF2 and YF5 lines.

(a) Adaxial epidermal layer of the third leaf of non-stressed WT (A), CDC2aWT (C), YF2 (E), YF5 (G) and stressed WT (B), CDC2aWT (D), YF2 (F), YF5 (H) plants

(b) Decrease of total surface of the third leaf upon salt stress

(c) Epidermal cell number per leaf area unit in normal and stress conditions

(d) Mean cell size of the third leaf in normal and salt stress conditions

(e) Elongation rates of hypocotyl upon salt stress

Fig. 3 Histone H1 CDK activity of stressed and non-stressed WT, CDC2aWT and YF2 lines.

Fig. 4 Percentage of G2 *Arabidopsis* cells with a 4C content with and without addition of NaCl to the medium (---. 0% NaCl; — 0.5% NaCl).

The present invention is further illustrated by reference to the following non-limiting examples.

Unless stated otherwise in the examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook et al. (1989), Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy,

jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Morphological alterations in response to salt stress and correlation to the expression of cell cycle regulatory genes

In order to investigate morphological alterations in response to salt stress, an histochemical analysis of three known plant cell cycle regulating proteins was performed. Therefor, time course experiments were performed on transgenic plants transformed with cyclin (CycA2;1, CycB1;1) and CDK (CDC2aAt) promoter-gus fusions respectively. Both cyclin and CDK promoters originated from *Arabidopsis thaliana*. Ten days old Arabidopsis plants were transferred to solid media containing 1% NaCl ; GUS activity and morphological changes were observed after 12hrs, 36hrs. 4 days, 1 week and two weeks. After 12hrs treatment the promoter activities declined in the apical meristem, before any morphological change was visible. After 36 hrs growth in the presence of salt the swollen roottips showed a decrease in expression of all cell cycle genes concomitant with a shrinkage of the root apical meristem. After four days, morphological alterations in the aerial part of the stressed plant were clearly visible when compared to control. The stressed plants were shorter due to a less elongated hypocotyl and the leaves were smaller. During adaptation to stress an induction of CycA2;1 and CycB1;1 expression in the shoot apical meristem could be noticed. Measurements of the length of the leaves and of the length of the meristematic region in the roots made after two weeks growth on salt containing medium, demonstrated a strong reduction in comparison to control plants. The number of leaves initiated was significantly lower in salt stressed plants than in control plants. In expanding leaves of salt stressed plants no GUS staining for CycB1;1 nor CDC2aAt expression could be detected contrarily to control plants, illustrating the decline in mitotic activity in these organs.

Example 2: Improved tolerance to salt stress of Arabidopsis thaliana containing a CDC2a-Y15F/T14A mutant gene under the control of a CaMV35S promoter

A comparative study was made between transgenic Arabidopsis plants containing a CDC2a-Y15F/T14A mutant gene under the control of a CaMV35S promoter (see Hemerly, EMBO J. 14 (1995), 3925-3936) and wild-type plants, in response to salt stress. Arabidopsis plants (ecotype C24) were engineered containing a mutated form of the CDC2aAt gene in which the phosphorylation sites T14 (Threonine at amino acid position number 14) and the Y15 (Tyrosine at amino acid position number 15) were changed in A14 (Alanine at amino acid position number 14) and F15 (Phenylalanine at amino acid position number 15) under control of a CaMV35S promoter. The overexpression of mutant CDC2a-Y15F/T14A in Arabidopsis lines did not show drastic changes in development. Only a tendency for loss for apical dominance could be noticed. Two independent transgenic *Arabidopsis* lines (YF2 and YF5) overexpressing mutant CDC2aAt with non-phosphorylatable Ala14 and Phe15 residues CDC2a-Y15F/T14A were selected to study their response on salt stress. As controls non-transformed Arabidopsis plants (C24) and transgenic plants carrying a construct of a non-mutated CDC2aAt gene under the control of a CaMV35S promoter were included in the experiment. Plants of ten days old, grown on solid germination medium, were transferred to the same medium containing 1% NaCl and their further growth and development was observed compared to the control plants. Both mutant lines displayed an improved tolerance to salinity which was phenotypically visible. The salt stressed mutant plants had bigger and more elongated leaves than control non transformed plants and plants overexpressing the wild type CDC2aAt gene. Furthermore, compared to wild type plants (WT) and transgenic *Arabidopsis* plants ectopically expressing the wild type form of CDC2aAt (CDC2aWT), the YF lines displayed an enhanced shoot growth after cultivation in the presence of NaCl. Additionally the YF lines recovered faster upon release from salinity than the CDC2aWT and WT plants (Fig. 1). As mentioned before, CDC2aWT and YF lines contain respectively the wild type and mutated CDC2aAt form in which Thr14 and Tyr15 were substituted for Ala14 and

Phe15 residues, placed under control of a constitutive CaMV 35S promoter (Hemerly, EMBO J. 14 (1995), 3925-3936). *Arabidopsis* seedlings (ecotype C24), grown for ten days in sterile conditions on solid K1 germination medium (Valvekens, Proc. Natl. Acad. Sci. USA 85 (1988), 5536-5540), were transferred for seven days to the same medium to which 1% NaCl was added. To release them from salinity they were transferred again to a solid K1 medium without NaCl. The picture was taken after seven days recovery.

To quantify growth, leaf epidermal cell numbers and hypocotyl lengths were measured. Ten days old *Arabidopsis* seedlings, grown in vitro on solid K1 medium, were transferred to the same medium with or without the addition of 1% NaCl respectively for seven days. Measurements of epidermal cell numbers and individual cell surfaces were obtained from digitalized camera-lucida drawings made from the adaxial leaf surface of the third leaf, using DIC optics on a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). The third leaf was chosen based on the expression of the *CycB1;1:gus* marker, representative for actively dividing cells (Ferreira, Plant Cell 6 (1994), 1763-1774), at the moment of transfer to the saline environment. A monolayer of epidermal cells was visualized in whole mounted leaves that had been fixed in 100% methanol, and cleared in lactic acid. Image analyses were performed with the public domain Scion Image Program (version ß-3b, Scion Corporation). The reported means for each genotype are the mean value of three independent measurements from three different leaves. Hypocotyllengths of at least ten plants per genotype were measured by means of a Zeiss stereomicroscope. All data were incorporated in histograms by use of the Excell Microsoft Program. In all tested lines, salt stress caused a decrease in the total leaf area and epidermal cell number (Fig. 2a,b). Interestingly, the epidermal cell density was significantly higher in YF2 and YF5 leaves (Fig. 2c), implying that more cell divisions had occurred during stress. In agreement, the mean epidermal cell size was smaller in YF than in WT and CDC2aWT lines (Fig. 2d), comparable to the yeast wee phenotype. Moreover, the stomatal complexes, formed by division from meristemoids (Yang, Plant Cell 7 (1995), 2227-2239), were more developed in stressed YF than in control lines (CDC2aAtWT and WT)

(Fig. 2a). Also the hypocotyl growth was reduced after transfer of the seedlings to a saline environment in WT and CDC2aWT lines while this was not the case in the YF lines (Fig. 2e). These data suggest that, contrarily to WT and CDC2aWT, elongation is not inhibited or retarded upon salt stress in the YF lines since cell divisions are not significantly involved in hypocotyl growth of *Arabidopsis* seedlings (Gendreau, Plant Physiol. 114(1) (1997), 295-305).

In order to correlate the observed phenotypes with CDC2 activity, the H1 kinase activities of the CDK complexes were determined (Fig. 3). *Arabidopsis* seedlings were grown for ten days in sterile conditions on filters on solid K1 medium and subsequently transferred to liquid K1 medium for two hours. The plants were then transferred to fresh liquid K1 medium with or without the addition of 1% NaCl. Samples were taken after 3 and 24hrs and H1 kinase activities were measured (Azzi, Eur. J. Biochem. 203 (1992), 353-360). Protein concentrations were determined using the Protein Assay kit (Bio-Rad, Munich, Germany), using Bovine Albumin Serum as a standard. Histone H1 kinase assays were performed with the CDK complexes purified from crude extracts by p13SUC1 affinity (Azzi, Eur. J. Biochem. 203 (1992), 353-360), using 50μg of total proteins and 20μl of a 50% suspension of $p13^{SUC1}$-agarose beads. Phosphorylated histone H1 was visualized through phosphorimager scanning (Molecular, Eugene, OR). CDC2 like kinase activities were rapidly decreasing upon salt stress in WT, but remained high in the CDC2aWT and YF2 lines. The growth discrepancies between the CDC2aWT and YF plants upon salinity were however not reflected in the kinase activity measurements. The quantitative detection limit of the method used for the determination of the kinase activities (Azzi, Eur. J. Biochem. 203 (1992), 353-360), may be restrictive to visualize the differences in kinase activities between the transgenic lines.

Hence, the enhanced growth of the YF plants demonstrates the importance of a regulatory control mechanism upon abiotic stress such as salt stress that inhibits CDC2aAt activity by alteration of phosphorylation status of the CDC2aAt complex. The activity of CDC2aAt is maximal at the G1/S and G2/M transitions, suggesting a functional involvement at both checkpoints.

In order to find out at which transition point this stress-induced control mechanism may be operative, the nuclear content of *Arabidopsis* cell suspensions was analyzed after addition of NaCl. The *Arabidopsis* cell line (Axelos, Mol. Gen. Genet. 219 (1989), 106-112) was subcultured every seven days in Gamborg B5 medium (Sigma) supplemented with 0.2 mg/l α-naphtalenic acid. NaCl (0.5 %) was added 48hrs after subculturing in fresh medium. To determine the DNA nuclear content of the cells, 1ml of the cell suspensions was centrifuged and the nuclei were released (Glab, FEBS Lett. 353(2) (1994), 207-211) before flow cytometry analysis (Biorad, Bryte HS). It was found that salinity caused a G2 phase arrest in *Arabidopsis* cell suspensions cultures (Fig. 4). A G2/M arrest has also been reported in dehydrated wheat leaves (Schuppler, Plant Physiol. 117 (1998), 667-678) and in yeast upon hyperosmolarity (Shiozaki and Russel, Nature 378 (1995), 739-743). In wheat leaves Tyr phosphorylation of CDC2 like proteins has been speculated (Schuppler, Plant Physiol. 117 (1998), 667-678) as a consequence of water stress but no clue whatsoever has been made about specific CDC2 proteins nor phosphorylation sites involved in this process.

As has been demonstrated above, for the first time genetic evidence is provided for a link between the regulation of cell cycle progression and growth inhibition by abiotic stress, in particular salt stress. The data obtained in accordance with the present invention demonstrate that plants have conserved the CDC2 Tyr15 checkpoint control at the G2/M border coupled to environmental signals. Mammals contain at least three distinct CDC25 Tyr15 phosphatases of which two are functionally implicated in the G2/M transition (Sadhu, Proc. Natl. Acad. Sci. USA 87 (1990), 5139-5143; Galaktionov, Cell 67 (1991), 1181-1194. Cell 57, 1181-1194; Nagata, New Biol. 10 (1991), 959-968; Suto, EMBO J. 13 (1994), 1549-1556). In fission yeast MIK1 acts cooperatively with the WEE1 protein kinase in the inhibitory Tyr15 phosphorylation of CDC2 (Lundgren, Cell 64 (1991), 1111-1122). Besides WEE1, a MYT1 protein kinase that phosphorylates CDC2 on both Thr14 and Tyr15, has been discovered in *Xenopus* (Mueller,

Science 270 (1998), 86-89). Considering the evolutionary conservative character of the cell cycle regulation, similar phosphatase and kinase relatives are expected to exist in plants. Genetic manipulation of these protein phosphatases and kinases that regulate CDK activity might contribute to engineer abiotic stress tolerant an in particular osmotolerant plants in the future.

In summary, the experiments performed in accordance with the present invention demonstrate that a stress-induced phosphorylation of a cyclin dependent kinase results in an inhibitory growth response. As has been discussed in the embodiments hereinbefore, this finding opens up the way for several beneficial applications in plant science and agriculture.

Example 3: Experimental setup to define other stress conditions

To verify if plants are also responding differently to other environmental stresses than salinity stress, experiments can be designed by the person skilled in the art in accordance with methods known in the art (see, e.g., references cited hereinbefore), for example exposure of the plants to

- Cold stress: 2-5°C
- Heat stress: 28-40°C
- Drought stress: withholding water for 5-14 days or withhold water for 5 days, supply water for 2 days and withhold water for another 5 days
- Freezing stress: -6 to –4°C
- Growth under stress conditions: Transfer of ten-days-old *Arabidopsis* seedlings (WT, *CDC2aAt*WT, YF2 and YF5) grown in sterile conditions on solid K1 medium to a growth chamber where the temperature is lowered to, for example, 4°C (cold shock) or increased to 28°C (heat shock). Observations can be made daily after 4 to 10 days transfer to observe growth differences.
- Recovery of stress conditions: Release of stressed plants (cold or heat shock) described above after 4 to ten days to normal growth conditions. Observations can made daily up to 15 days after the moment of release.

The period of exposure to a stress and the empirical values of the stress (e.g., 40°C vs. 6°C) will depend upon the species of plant being tested, however, a person skilled in the art is able to easily determined these periods or values.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated by reference.

CLAIMS

1. A method for obtaining plants, tolerant to abiotic stress conditions, comprising introducing into a plant cell, plant tissue or plant a nucleic acid molecule or regulatory sequence, wherein the introduction of said nucleic acid molecule or regulatory sequence results in the presence of a Cyclin Dependent Kinase (CDK) protein that is not susceptible to inhibitory phosphorylation under abiotic stress conditions.

2. The method of claim 1, wherein said CDK is a PSTAIRE type CDK.

3. The method of claims 1 or 2, wherein said CDK is CDC2a.

4. The method of any one of claims 1 to 3, wherein said CDK is derived from *Arabidopsis thaliana*.

5. The method of any one of claims 1 to 4, wherein the CDK is free of phosphate at the tyrosine at a position that corresponds to position 15 in the amino acid sequence of CDC2a of *Arabidopsis thaliana*.

6. The method of any one of claims 1 to 5, wherein the CDK protein is free of phosphate groups at both the tyrosine and the threonine, corresponding to the tyrosine at position 15 and the threonine at position 14, respectively, in the amino acid sequence of CDC2a of *Arabidopsis thaliana*.

7. The method of any one of claims 1 to 6, wherein said CDK protein is a non-phosphorylatable CDK mutein.

8. The method of claim 7, wherein the tyrosine at position 15 of said CDK mutein is substituted to a non-phosphorylatable amino acid residue.

9. The method of claim 8, wherein said CDK mutein further comprises a non-phosphorylatable amino acid residue at position 14.

10. The method of any one of claims 7 to 9, wherein the CDK mutein comprises a Y-15->F-15 mutation.

11. The method of any one of claims 7 to 10, wherein the CDK mutein also comprises a T-14->A-14 mutation.

12. The method of any one of claims 7 to 11, wherein said nucleic acid molecule encodes said non-phosphorylatable form of CDK.

13. The method of any one of claims 1 to 6, wherein said non-phosphorylated form of CDK is due to dephosphorylation and/or inhibition of phosphorylation of CDK.

14. The method claim 13, wherein said dephosphorylation is conferred by CDC25 or a functional analogue thereof, capable of dephosphorylation at least the tyrosine at position 15 of the endogenous CDK of said plant.

15. The method of claim 13, wherein said inhibition of phosphorylation is conferred by the suppression of expression or activity of Wee-kinase, MIK, MYT or a functional equivalent thereof, inhibiting the endogenous phosphorylation of at least the tyrosine at position 15 of the CDK of the said plant.

16. The method of claim 14 or 15, wherein said nucleic acid molecule encodes said CDC25, Wee-kinase MIK, MYT or functional analogue or equivalent thereof.

17. The method of any one of claims 1 to 16, wherein said nucleic acid molecule is operatively linked to regulatory sequences allowing the

expression of the nucleic acid molecule in the plant cell.

18. The method of any one of claims 1 to 17, wherein the regulatory sequence comprises a promoter, enhancer, silencer, intron sequence, 3'UTR and/or 5'UTR region, protein and /or RNA stabilizing elements.

19. The method of any one of claims 1 to 18, wherein said regulatory sequence is a chimeric, tissue specific, constitutive or inducible promoter.

20 The method of claim 19, wherein said inducible promoter is inducible by abiotic stress.

21. The method of 20, wherein said abiotic stress is osmotic stress.

22. The method of any one of claims 1 to 21, wherein said plant is a monocotyledonous or a dicotyledonous plant.

23. The method of any one of claims 1 to 22 wherein said plant is a crop plant, root plant, oil producing plant, wood producing plant, agricultured bioticultured plant, fruit producing plant, fodder or forage legume, companion plant or horticultured plant.

24. The method of claim 22 or 23, wherein said plant is wheat, barley, maize, rice, carrot, sugar beet, chicory, cotton, sunflower, tomato, cassava, grapes, soybean, sugar cane, flax, oilseed rape, tea, canola, onion, asparagus, carrot, celery, lentil, broccoli, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, rye, sorghum, oats, tobacco, pepper, grape or potato.

25. A vector comprising the nucleic acid molecule as defined in claim 20 or 21.

26. A transgenic plant cell comprising at least one nucleic acid molecule as

defined in claim 20 or 21 or a vector of claim 25.

27. A transgenic plant cell comprising at least one nucleic acid molecule or regulatory sequence as defined in any one of claims 1 to 21 or a vector of claim 25 and comprising a further nucleic acid molecule that is capable of conferring to a transgenic plant an additional phenotypic characteristic.

28. A transgenic plant or plant tissue comprising plant cells of claim 26 or 27.

29. The transgenic plant of claim 28 which displays increased tolerance to abiotic stress, preferably osmotic stress, compared to the corresponding wild type plant.

30. The transgenic plant of claim 29 which displays an additional phenotypic characteristic.

31. Harvestable parts or propagation material of a plant of any one of claims 28 to 30 comprising plant cells of claim 26 or 27.

32. Use of a nucleic acid molecule or regulatory sequence as defined in any one of claims 1 to 24 or a vector of claim 25, for conferring abiotic stress tolerance to a plant and/or as a selectable marker in plants.

33. Use of a nucleic acid molecule or regulatory sequence capable of counteracting stress-induced down-regulation of cell division for the production for osmotic, preferably salt stress tolerant plants.

34. Use of a plant obtainable by the method of any one of claims 1 to 24 or the plant of any one of claims 28 to 30 for culturing on soil with a salt content of about 40 mM to about 300 mM.

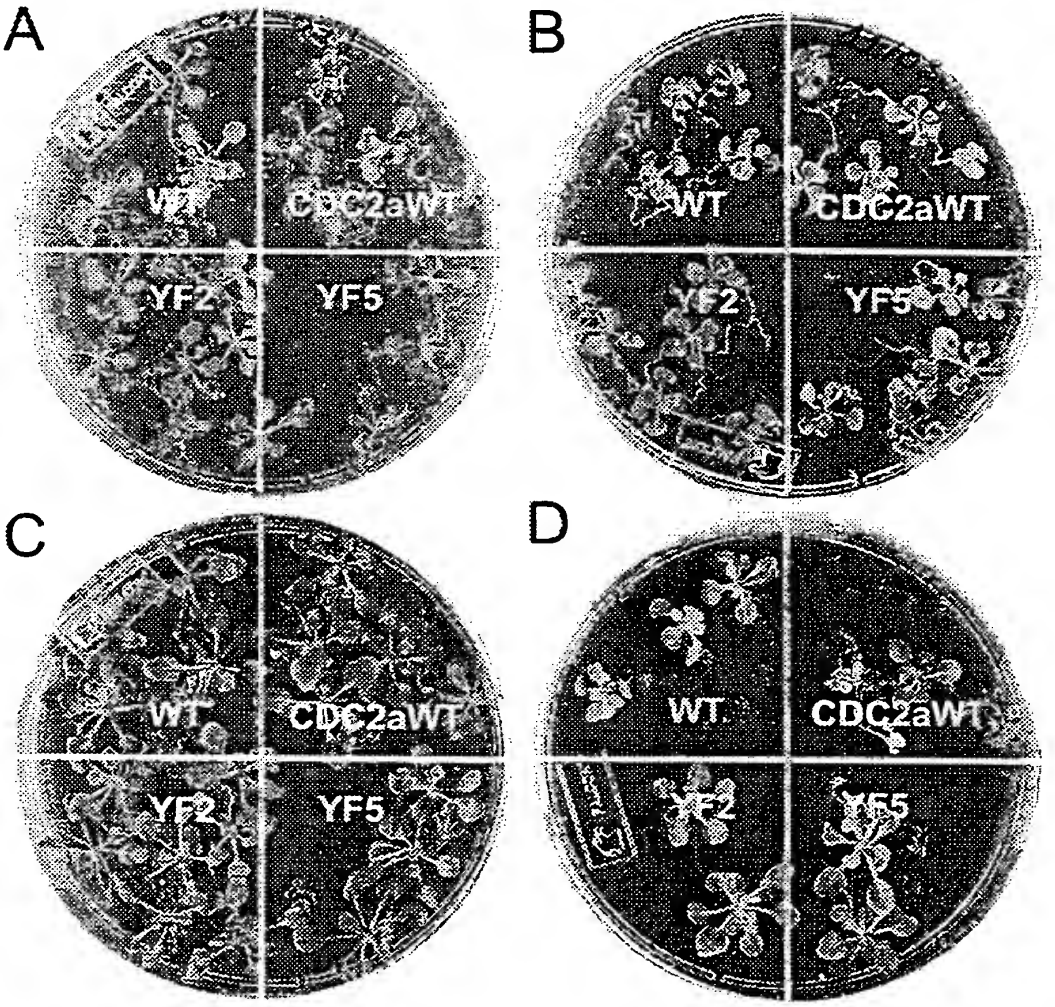

Fig. 1

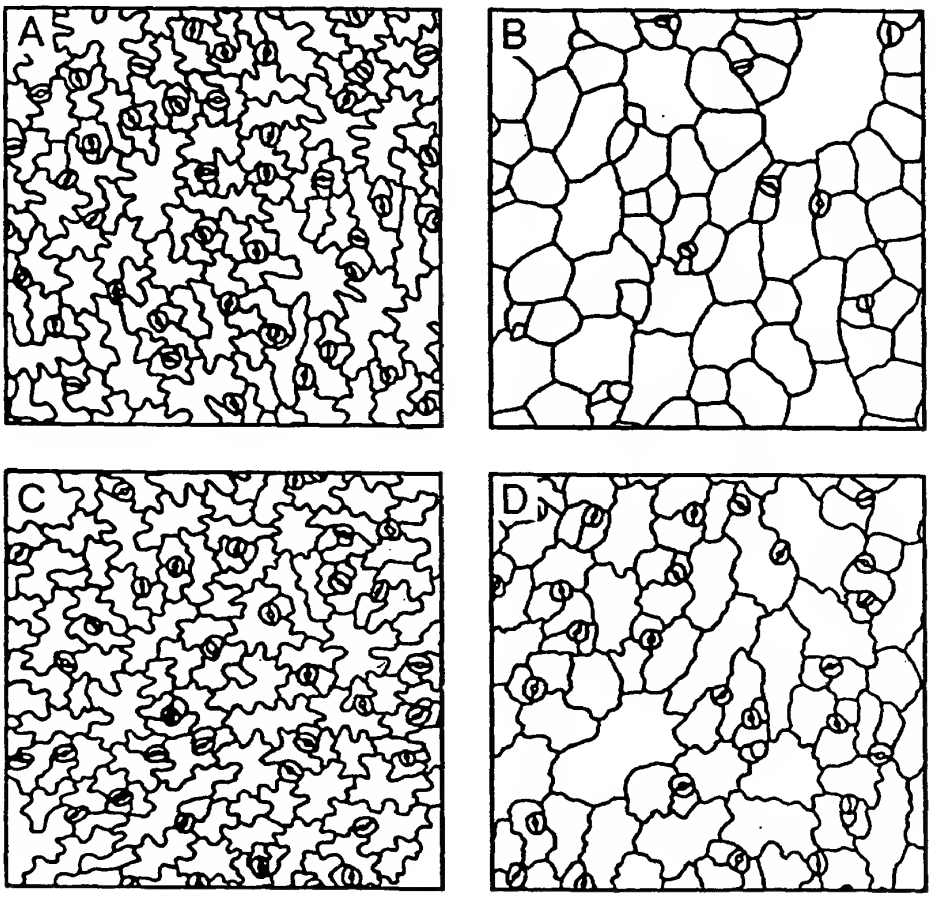

Fig. 2a

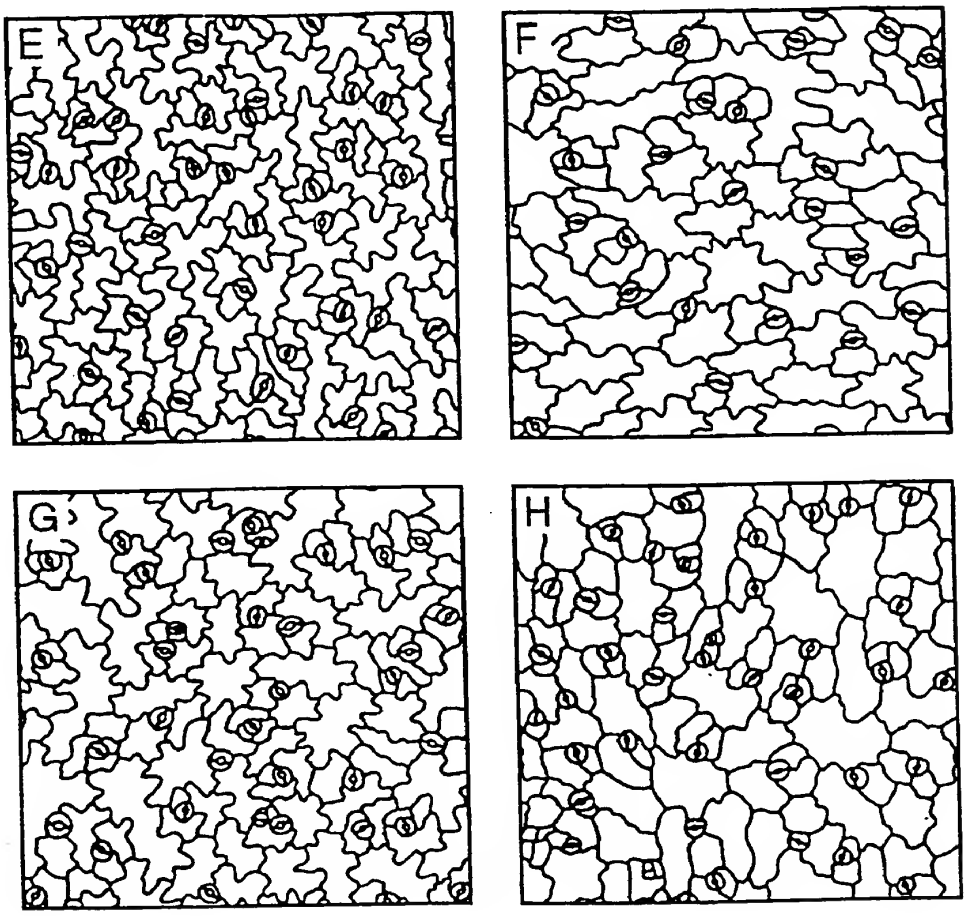

Fig. 2a continued

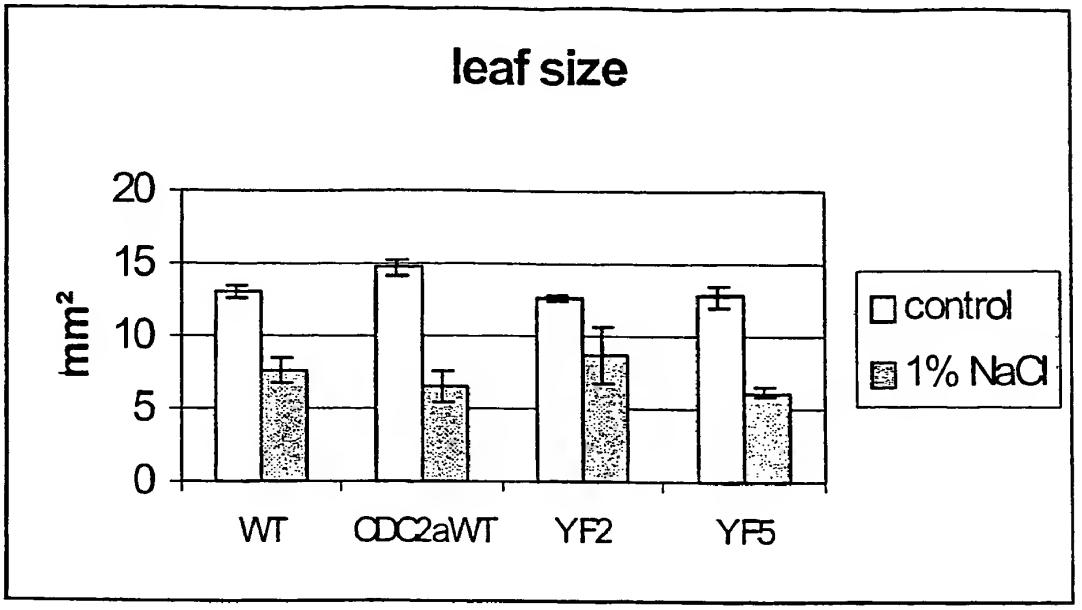

Fig.2b

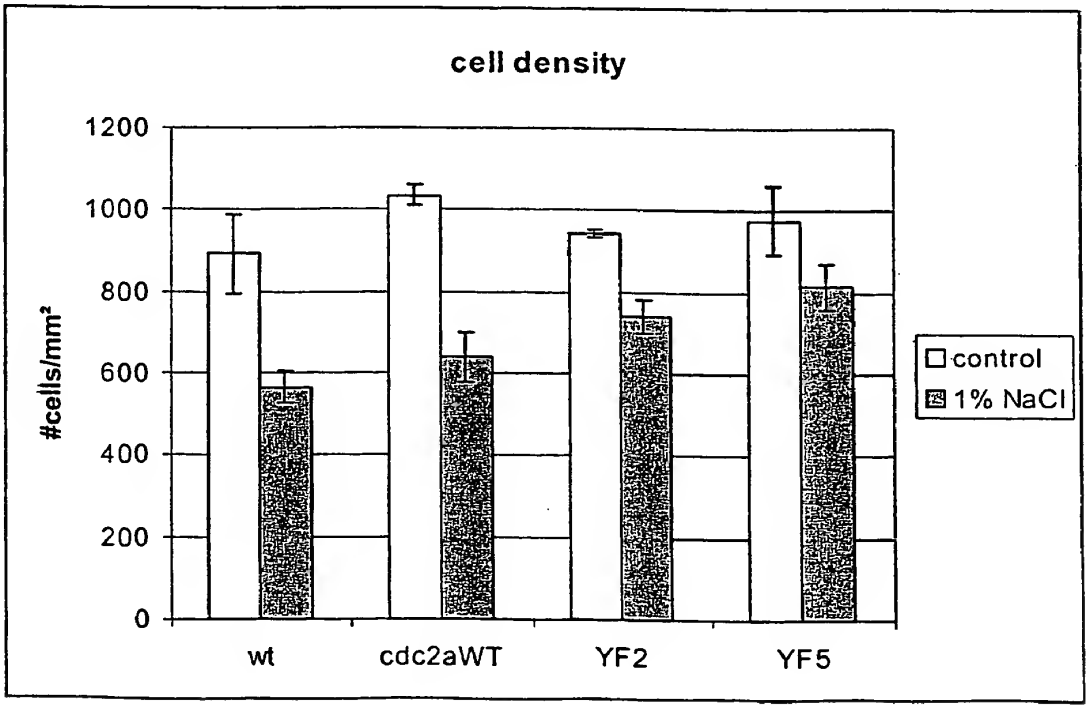

Fig.2c

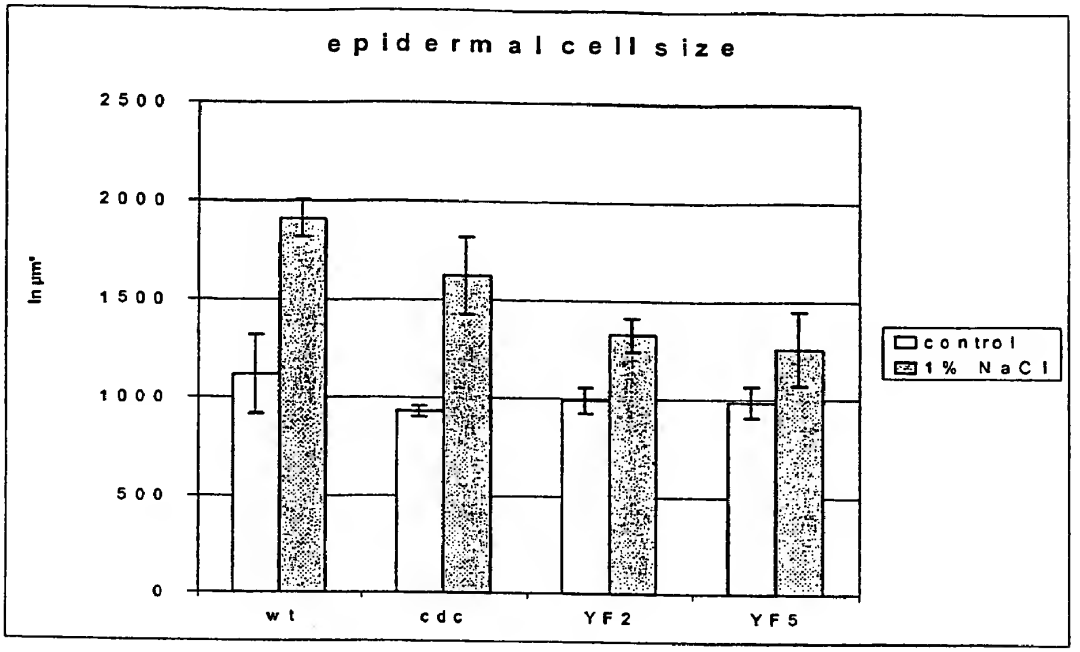
epidermal cell size
2500
2000
1500
1000
500
0
In µm²
wt
cdc
YF2
YF5
control
1% NaCl

Fig.2d

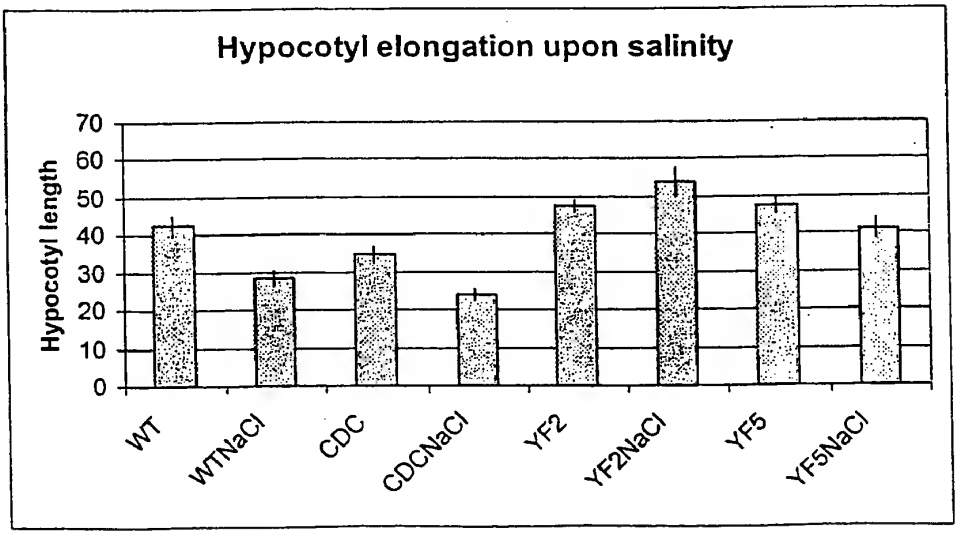
Hypocotyl elongation upon salinity
Hypocotyl length
70
60
50
40
30
20
10
0
WT
WTNaCl
CDC
CDCNaCl
YF2
YF2NaCl
YF5
YF5NaCl

Fig.2e

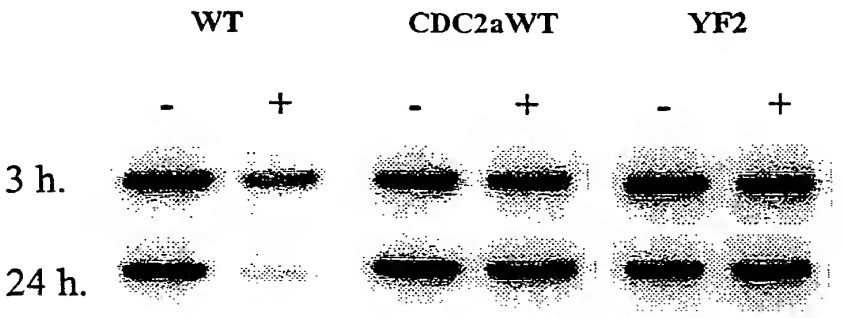

Fig. 3

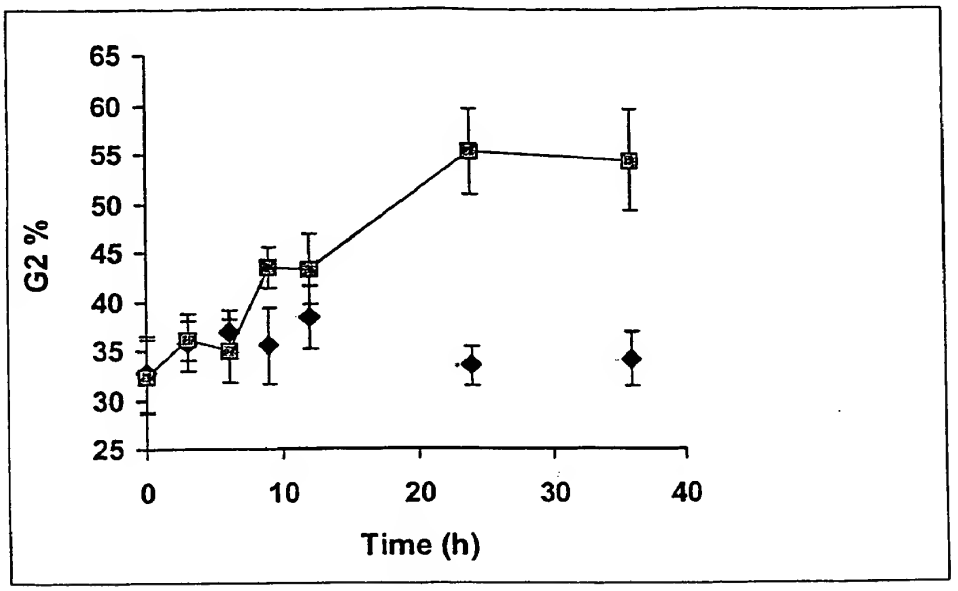

Fig. 4

INTERNATIONAL SEARCH REPORT

In ıtional Application No
PCT/EP 99/02696

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 09685 A (UNIV AUSTRALIAN) 11 June 1992 (1992-06-11) page 6, line 9 - line 32; claims 23-28 ---	25-34
X	DATABASE SCISEARCH 'Online! INSTITUTE FOR SCIENTIFIC INFORMATION, PHILADELPHIA, PA, US AN 96:404830, BARNA, B.: "Production of disease-tolerant and stress-tolerant plants by increasing their juvenility and antioxidant capacity" XP002080509 abstract & NOVENYTERMELES, vol. 44, no. 5/6, 1995, pages 561-567, --- -/--	33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1999	14/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/EP 99/02696

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X	BELL M H ET AL: "TOBACCO PLANTS TRANSFORMED WITH CDC25, A MITOTIC INDUCER GENE FROM FISSION YEAST" PLANT MOLECULAR BIOLOGY, vol. 23, 1993, pages 445-451, XP002045515 the whole document ---	27-31
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X	DE VEYLDER, L., ET AL.: "Expression of mutant CDC2aAt genes in plants with the use of chemical inducible promoters" MED. FAC. LANDNOUWW. UNIV. GENT, vol. 60/4a, 1995, page 1701 XP002080508 the whole document ---	25-31
P,X	WO 99 14331 A (ALMEIDA JANICE DE ;LANDRIEU ISABELLE (BE); VEYLDER LIEVEN DE (BE);) 25 March 1999 (1999-03-25) page 41, line 13 - line 24 page 42, line 1 - line 3; claim 41 ---	33
A	BRACALE, M., ET AL.: "Water deficit in pea root tips: effects on the cell cycle and on the production of dehydrin-like proteins" ANN BOT, vol. 79, 1997, pages 593-600, XP002080503 the whole document ---	1,32-34
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	NAGL, W.: "Cdc2-kinases, cyclins, and the switch from proliferation to polyploidization" PROTOPLASMA, vol. 188, 1995, pages 143-150, XP002076333 the whole document	1
A	NAGL,W.: "Induction of high polyploidy in Phaseolus cell cultures by the protein kinase inhibitor, K-252a" PLANT CELL REPORTS, vol. 12, 1993, pages 170-174, XP002076331	1
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A	WO 98 03631 A (SALK INST FOR BIOLOGICAL STUDI) 29 January 1998 (1998-01-29) the whole document	1
E	WO 99 22002 A (ALMEIDE JANICE DE ;VEYLDER LIEVEN DE (BE); INZE DIRK (BE); CROPDES) 6 May 1999 (1999-05-06) page 21 last 3 lines page 26, paragraph 3 page 32, line 9 - line 20; claims 18,28	33

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PCT/EP 99/02696

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